Estrogen-dependent responses of the mammary fat pad in prepubertal dairy heifers

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Abstract

Ovaries are absolutely required for development of the mammary parenchyma (PAR) in cattle, reflecting estrogen-dependent epithelial cell proliferation. However, the estrogen receptor (ER) that mediates the mammary estrogen effects, ERα, is absent in proliferating epithelial cells. In the mouse, this discrepancy is explained in part by the ability of the mammary fat pad (MFP) to synthesize epithelial cell mitogens such as IGF-I in response to estrogen. Consistent with a similar role for the bovine MFP, 30% of its fibroblasts and adipocytes were immunoreactive for ERα in prepubertal dairy heifers. To assess estrogen-dependent gene expression in the MFP, 16 prepubertal dairy heifers were randomly assigned to a 2×2 factorial. The first factor was ovarian status, with heifers undergoing bilateral ovariectomy or left intact at 4·6 months of age. The second factor was applied 30 days after surgery and consisted of injection of estrogen or excipient. After 3 days of injection, heifers were administered an intrajugular bolus of bromodeoxyuridine (BrdU) and slaughtered 2 h later. The estrogen injection, but not ovarian status, caused significant increases in the fraction of epithelial cells labeled with BrdU and produced tissue-specific effects on gene expression. In the PAR, estrogen injection increased IGF-I gene expression by twofold despite reductions of 50% or more in ERα mRNA abundance and the fraction of epithelial cells immunoreactive for ERα. The estrogen-dependent increase in IGF-I mRNA was greater in the MFP, presumably because estrogen failed to downregulate ERα expression in this mammary compartment. Finally, estrogen-responsiveness of the MFP appears unique among the bovine fat depots as estrogen injection did not induce IGF-I expression in its s.c. counterpart. Our data demonstrate that the bovine MFP is highly responsive to exogenous estrogen, consistent with a role for this tissue compartment in communicating its effects on epithelial cell proliferation.

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Introduction

In prepubertal cattle, development of the mammary parenchyma (PAR) is severely impaired when ovaries are removed soon after birth (Wallace 1953, Purup et al. 1993, Berry et al. 2003b). This impairment is caused, at least in part, by a lower rate of mammary epithelial cell proliferation in ovariectomized heifers (Berry et al. 2003b). Conversely, prepubertal mammary development in ovariectomized heifers is restored by the administration of an estrogenic compound (Wallace 1953). These observations suggest that the effects of ovaries on the developing PAR are mediated by estrogen.

The estrogen effects are mediated predominantly by the estrogen receptor α (ERα) in the mammary gland (Couse & Korach 1999, Connor et al. 2005). Paradoxically, most bovine mammary epithelial cells that proliferate in response to exogenous estrogen do not express ERα (Capuco et al. 2002).

This lack of overlap between ERα expression and proliferation has also been observed in the rat, mouse and human mammary epithelial cells (Zeps et al. 1998, Russo et al. 1999, Saji et al. 2000). These observations led to the hypothesis that non-epithelial, ERα-positive cells play an important role in mediating estrogen effects. According to this model, these cells respond to estrogen by secreting growth factors, which stimulate proliferation of neighboring, ERα-negative epithelial cells. A well-studied example of such a mammary epithelial cell mitogen is insulin-like growth factor-I (IGF-I; Shamay et al. 1988). IGF-I is synthesized exclusively by non-epithelial cells in the bovine PAR (Plath-Gabler et al. 2001, Berry et al. 2003a) and its transcription is increased by estrogen (Umayahara et al. 1994).

Most studies in cattle have been largely epitheliocentric and have not considered the possibility that the mammary fat pad (MFP) plays a role in mediating the effects of estrogen on

the PAR (Akers *et al.* 2005). The bovine MFP consists predominantly of adipocytes and fibroblasts, and both cell types have been shown to account for most of the non-epithelial, ER-positive cells required for normal epithelial development in the prepubertal mouse (Cunha *et al.* 1997). Finally, estrogen administration to prepubertal cattle stimulates epithelial cell proliferation to the greatest extent in the parenchymal region adjacent to the MFP (Capuco *et al.* 2002). These observations suggest that the bovine MFP could be involved in communicating the influence of estrogen on the developing PAR. Finally, the possibility that the MFP responds differently to estrogen than s.c. adipose tissue (SQA), as shown in rodents (Walden *et al.* 1998), has not been examined in cattle.

To address these questions, we asked whether significant levels of immunoreactive ERα were present in the MFP. We then varied plasma estrogen levels by subjecting prepubertal heifers to bilateral ovariectomy (OVX) and exogenous estrogen administration, and examined the expression of IGF-I and progesterone receptor (PR) genes in MFP, PAR and SQA. Our data show that the MFP expresses significant levels of ERα and has a greater ability to produce IGF-I in response to estrogen than either the PAR or SQA.

Materials and Methods

Animals

The Cornell University Animal Care and Use Committee approved all the procedures used in this study. To evaluate the presence of ERα in the MFP, six heifers were purchased from commercial dairy farms and moved at day 10 of life to the Cornell Research Center. They were offered milk replacer until weaning (8·4 weeks) and a complete total ration thereafter at rates needed to sustain an average daily gain of 650 g/day. Heifers were slaughtered when their body weight was 150 kg, by stunning with a captive bolt and exsanguination. The mammary gland was removed immediately and split into right and left halves. Tissues were dissected from the mid-MFP and fixed overnight in 10% neutral buffered formalin at 4 °C and stored in 70% ethanol.

To assess the effects of estrogen on gene expression, 16 prepubertal Holstein heifers were purchased at 3·2 months of age from a single commercial dairy farm and transported to the Cornell Research Center. Thereafter, they were fed a complete total ration at rates needed to sustain an average daily gain of 650 g/day. After an acclimatization period of 45 days, they were randomly assigned to a 2×2 factorial. The first factor was ovarian status, with eight heifers each undergoing bilateral OVX at 4·6 months (106 kg), or left intact (INT). Ovaries were accessed via a laparoscopic technique under halothane anesthesia and removed using diathermy methodology. The second factor was applied after a surgery recovery period of 30 days and consisted of daily s.c. injection of 17β-estradiol (+estrogen) or excipient (corn oil,

−estrogen). Estrogen was administrated at a dose of 0·1 mg/kg body weight as previous work has demonstrated that this dose elicits an increase in mammary epithelial cell proliferation in the prepubertal bovine (Woodward *et al.* 1993, Berry *et al.* 2001, Capuco *et al.* 2002). Injections were administered on 3 consecutive days. Approximately, 4 h after the last injection, heifers were given an intrajugular dose of bromodeoxyuridine (BrdU, 5 mg/kg body weight) as described previously (Capuco *et al.* 2002). Heifers were killed 2 h later (or 54 h after initial injection) by captive bolt stunning and exsanguination. The mammary gland was removed and weighed. Mid-PAR and MFP were obtained from the left half of the mammary gland and SQA was obtained from the brisket region. All tissues were snap frozen in liquid nitrogen for later RNA isolation. Additional PAR and MFP samples were fixed overnight in 10% neutral buffered formalin at 4 °C and stored in 70% ethanol until processed for immunohistochemistry.

Following tissue collection, the gland was skinned and separated into right and left halves at the medial suspensory ligament. The skin and teats from the whole gland were weighed together and the skinned right half was weighed separately. The weight of the skinned left half was determined by difference. The cervix, uterus, and uterine horns were excised and weighed and at this time, successful OVX was confirmed in all eight OVX heifers by visual appraisal.

Jugular blood was collected daily at 0700 h during the 8-day period preceding OVX and again between days 7 and 14 following surgery. During the injection period, blood was collected at 0, 12, 24, 36, and 48 h after the initial injection. Plasma was prepared by centrifugation and assayed for 17β-estradiol with a commercial RIA kit (Estradiol Maia, Adaltis Italia S.p.A., Casalecchio di Reno, BO, Italy) as described previously by Beam & Butler (1997).

Immunohistochemistry

Immunohistochemical detection of ERα and BrdU was performed exactly as described previously by bright field microscopy (Capuco *et al.* 2002). Briefly, the primary antibody for ERα localization was mouse monoclonal antibody C-311 produced against amino acid sequence 405–595 of the bovine ERα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and the primary antibody for BrdU was mouse monoclonal antibody clone BMC 9318 (Chemicon International, Inc., Temecula, CA, USA). To quantify the number of ERα and BrdU labeled cells, photographs of stained sections were captured as digital images with a Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) on a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY, USA) at a magnification of 400×. For each antibody, ten random areas were photographed from a single stained slide containing 3–4 sections. Labeled cells and total cells per micrograph were counted manually. A minimum of 4000 cells per animal was scored for each immunohistochemical analysis.

RNA isolation and quantitative real-time reverse transcriptase (RT)-PCR

Total RNA was isolated using the RNeasy Lipid Tissue Mini kit with on-column DNase digestion (Qiagen). Quality and quantity of the DNase-treated RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). DNase-treated RNA (990 ng) was reverse transcribed using the iScript cDNA Synthesis Kit as recommended by the manufacturer (Bio-Rad Laboratories). Transcript abundance was determined by SYBR green quantitative real-time RT-PCR (iQ SYBR Green Supermix; Bio-Rad Laboratories). A total of $1 \cdot 0$ µl of the RT reaction was used in the 25 µl PCR. Primers and PCR conditions for evaluation of ERα, ERβ, PR, and estrogen-related receptor α (ERRα) were as described (Connor *et al.* 2005). Forward and reverse primers for evaluation of IGF-I gene expression were 5′-CATCCTCCTCGCATCTCTTC-3′ and 5′-GCACAC-GAACTGGAGAGC-3′ respectively ($T_m = 57 \cdot 0$ °C; 118-bp product). Amplicons were verified for each assay by sizing on an Agilent 2100 Bioanalyzer and sequencing. Sample concentrations were determined from a standard curve of known target cDNA copy numbers ($1 \times 10^2 - 1 \times 10^7$ molecules).

Statistical analysis

Data were analyzed by a mixed model accounting for ovarian status (ovary, OVX, or INT), 17β-estradiol injection (estrogen, −estrogen, or +estrogen) and their interaction as fixed effects, and heifer as the random effect. To compare estrogen responsiveness across tissues, a fractional estrogen response was calculated for the IGF-I and PR data as follows. The copy number for each estrogen-treated heifer was subtracted from basal copy number and expressed as percentage of basal copy number. The basal copy number corresponds to the average of all oil-treated heifers. These data were analyzed by a mixed model accounting for ovarian status (ovary, OVX, or INT), tissue (PAR, MFP, or SQA) and their interaction as fixed effects, and heifer as the random effect. Unless otherwise mentioned the level of statistical significance was set at $P < 0 \cdot 05$.

Results

Immunodetection of ERα in the MFP

We recently reported negligible ERβ, but significant ERα, expression in MFP of prepubertal dairy heifers (MJ Meyer, RP Rhoads, AV Capuco, EE Connor, YR Boisclair and ME Van Amburgh, unpublished observations). The expression of ERα in the MFP was not obvious, given that it was found nearly exclusively in epithelial cells in the PAR (Capuco *et al.* 2002). To determine if ERα is expressed in the MFP, we performed immunohistochemistry on an initial

group of prepubertal heifers when weighing 150 kg. The ERα signal was clearly seen and localized to $33\% \pm 6$ of all fibroblasts and $31\% \pm 3$ of all adipocytes. A similar result is evident in micrographs obtained in the INT heifers used to evaluate estrogen effects in the MFP (Fig. 1, MFP, row A).

### Effect of estrogen status on plasma estrogen and tissue weight

To compare estrogen effects in PAR, MFP, and SQA, we used ovariectomized and intact heifers treated with or without exogenous estrogen. Prior to OVX, the plasma estrogen concentration fluctuated between the detectable limit of $0 \cdot 06$ pg/ml and approximately $4 \cdot 5$ pg/ml in all heifers. A week after surgery, the plasma estrogen concentration dropped to near or below the detectable limit in OVX heifers. Within 12 h of the first estrogen injection, the plasma estrogen concentration reached an average of 385 pg/ml in both OVX and INT heifers and stayed at this level for the duration of the injection period. Therefore, treatments caused expected changes in plasma estrogen.

Next, we assessed the effectiveness of treatments on tissue growth. Body weight at slaughter was not affected by ovarian status or estrogen injection. Short-term estrogen administration had no effect on PAR weight but caused a 79% increase in uterine weight (Fig. 2, $P < 0 \cdot 05$). OVX tended to reduce uterine weight by 31% (Fig. 2, $P < 0 \cdot 14$), but only led to a non-significant 23% reduction in PAR. To obtain a dynamic measure of treatment effects on the mammary gland, we quantified the percentage of PAR epithelial cells labeled after bolus administration of the thymidine analog BrdU. OVX elicited a non-significant 30% reduction in the percentage of BrdU-labeled epithelial, whereas estrogen injection increased labeling by over threefold irrespective of the ovarian status (Fig. 3, $P < 0 \cdot 05$). Overall, these data show that increased plasma estrogen has functional consequences on mammary epithelial cells.

Effect of estrogen on gene expression in PAR, MFP, and SQA

Ovarian status had no effect on expression of the ERα, ERRα, IGF-I, and PR genes in the PAR (Table 1). In contrast, exogenous estrogen caused a 50% reduction in ERα transcript abundance (Table 1, $P < 0 \cdot 01$). This reduction reached 80% when the response examined was the fraction of mammary epithelial cells displaying ERα immunoreactivity (Figs 1 and 4, $P < 0 \cdot 05$). Despite reduced parenchymal ERα expression, exogenous estrogen stimulated the expression of estrogen-responsive genes, causing a $1 \cdot 4$-fold increase in the case of IGF-I and a 5-fold increase in the case of PR (Table 1, $P < 0 \cdot 01$).

As seen in the PAR, estrogen injection caused gene expression responses in the MFP (Table 1). Estrogen injection caused a similar absolute increase in IGF-I transcript copy number in both INT and OVX heifers, and a greater increase in PR transcripts in OVX than in INT heifers (Ovary × estrogen, $P < 0 \cdot 01$). In contrast to what was seen in the PAR,
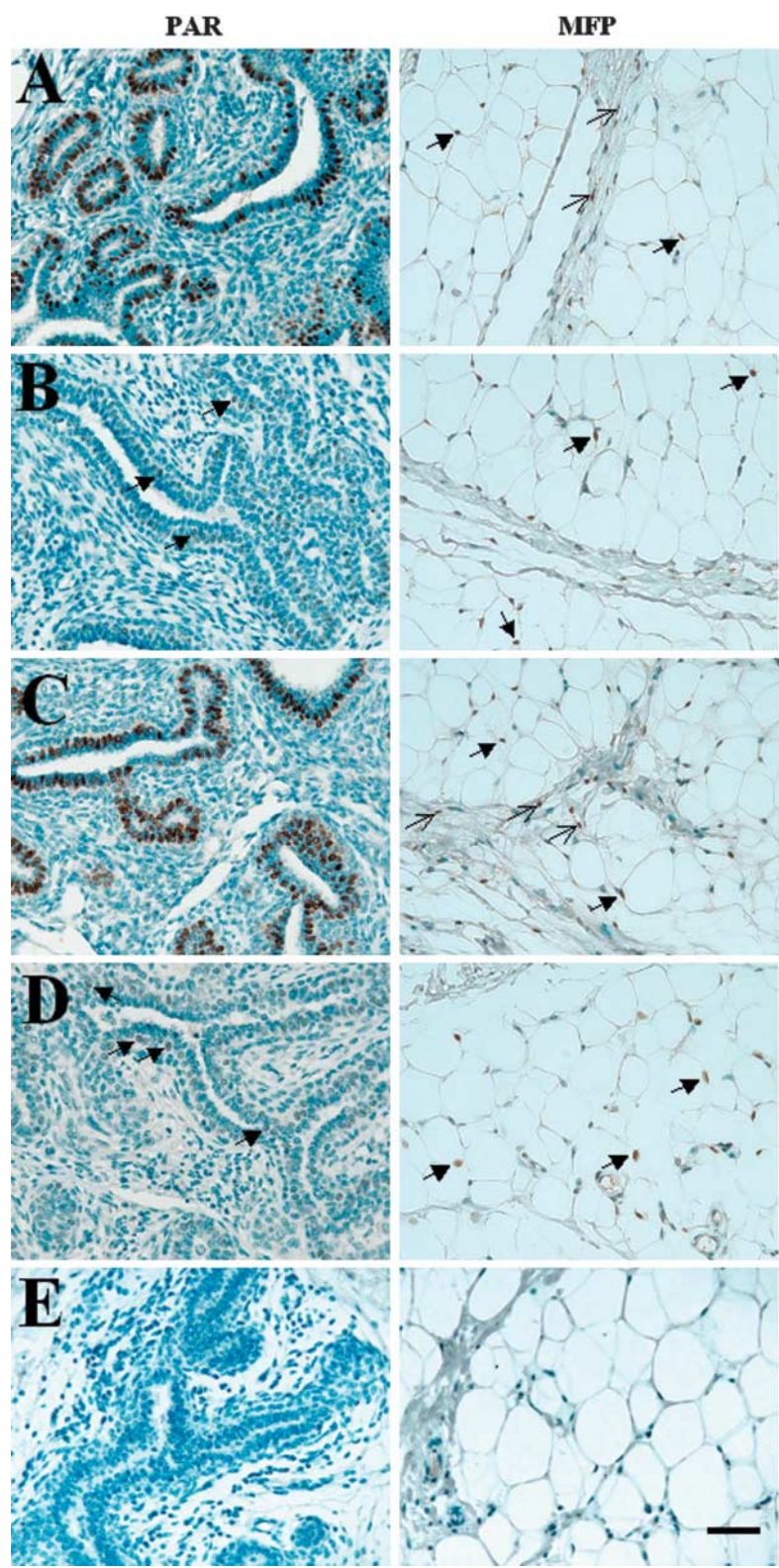
however, estrogen injection failed to reduce ERα transcript abundance in the MFP. Consistent with the mRNA result, estrogen injection did not induce a reduction in the number of cells displaying ERα immunoreactivity in the MFP of either INT or OVX heifers (Fig. 1).

Finally, SQA responses differed from the MFP and PAR in two ways. First, the SQA was the only tissue where both ovarian status and estrogen injection caused significant changes in ERα transcript abundance (31% increase in OVX and 32% reduction with estrogen injection, $P < 0.06$ or less; Table 1). Sensitivity of SQA to OVX was not explained by the presence of the other ER isoform (ERβ) as its expression was below our lowest standard (results not shown). Second, unlike MFP and PAR, the SQA did not increase IGF-I or PR gene expression in response to estrogen injection.

In the oil-treated heifers, absolute copy number for the IGF-I and PR transcripts differ 3 to 13-fold between PAR, MFP, and SCA, making the cross-tissue comparison of estrogen responsiveness difficult. To perform a relative comparison, copy number for each estrogen-treated heifer was subtracted from basal copy number and expressed as percentage of basal copy number. In this analysis, the basal copy number corresponds to the average of all excipient-treated heifers as the initial analysis indicated that ovarian status had no effect (Table 1). For IGF-I, the MFP was more estrogen responsive than the PAR, whereas the SQA completely failed to mount any estrogen-dependent response (Fig. 5, tissue, $P < 0.05$). For PR, the SQA also failed to respond, but only the MFP from INT heifers appeared to be more responsive than PAR (ovary × tissue, $P < 0.05$).

Discussion

Prepubertal mammary development is best understood in rodents (Hennighausen & Robinson 2005). In these animals, normal ductal growth requires presence of the MFP and estrogen (Cunha *et al.* 1997, Wiseman & Werb 2002). This dual requirement is explained in part by the ability of estrogen to induce the synthesis of growth factors in the MFP. These growth factors bind to their cognate receptors in epithelial cells resulting in cell proliferation. Recent experiments in genetically engineered mice have identified IGF-I as one of these growth factors. Briefly, ductal growth is impaired in IGF-I knockout (KO) mice even after exogenous estrogen administration (Ruan & Kleinberg 1999). IGF-I is required specifically by the epithelial compartment as shown by the failure of IGF-IR

KO epithelium to grow when grafted in a wild-type MFP (Bonnette & Hadsell 2001). Finally, the MFP produces IGF-I in an estrogen-dependent manner (Umayahara *et al.* 1994, Walden *et al.* 1998) and sustains normal ductal growth even when plasma IGF-I is reduced (Richards *et al.* 2004).

OVX early after birth also blocks mammary epithelial cell growth in cattle (Purup *et al.* 1993, Berry *et al.* 2003b). In their study, Purup *et al.* (1993) measured low plasma estrogen concentration (0·31 ng/ml) in prepubertal dairy heifers, raising the possibility that another ovarian factor was responsible for prepubertal development. Recently, however, Evans *et al.* (1994a,b) detected peaks in plasma estrogen concentrations in prepubertal heifers, ranging from 2·8 to 5·7 pg/ml, as early as 2 months of age and continuing to estrus. Consistent with their findings, the plasma estrogen concentrations in our intact heifers often exceeded 4 pg/ml. Circulating estrogen in prepubertal cattle reflects repeating waves of non-ovulatory follicular growth initiated approximately every 8 days (Evans *et al.* 1994a, b). These data strongly suggest that estrogen is the ovarian factor stimulating mammary epithelial cell growth in cattle.

The estrogen dose employed in the present study was chosen because an effect on mammary epithelial cell proliferation was assured (Woodward *et al.* 1993, Berry *et al.* 2001, Capuco *et al.* 2002), however, the effect on plasma estrogen levels was not previously described. It is difficult to discern potential differences in response to physiological versus pharmacological (as observed in the present study) plasma estrogen, as such data do not exist.

As in the case of rodents, data available in cattle support a model whereby estrogen does not exclusively act directly on the mammary epithelium. Within the PAR compartment, immunoreactive ERα localizes almost entirely to epithelial cells, but is virtually absent from the subset of those cells engaged in proliferation. These proliferating cells are found in highest density at the interface with the MFP in estrogen-treated heifers (Capuco *et al.* 2002), suggesting a role for the MFP in mediating or amplifying mitogenic estrogen effects. To examine whether the bovine MFP is a major site of estrogen action in the mammary gland, we ovariectomized heifers at 4·6 month of age and collected tissues 30 days later. OVX caused comparable non-significant reduction in the weight of the mammary PAR and uterus. Failure to detect an OVX effect on the weight of these tissues likely relates to the small number of animals in which this was evaluated. A second important factor, however, is the timing of the OVX procedure. In previous studies, OVX before 2·5 months of age nearly ablated PAR growth measured 3·5–6·5 months

Figure 1 Effect of ovarian status and 17β-estradiol injection on estrogen receptor α (ERα immunoreactivity in the prepubertal bovine mammary gland. Sixteen dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 months of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or +estrogen). Heifers were slaughtered after 3 days of injection and the mammary parenchyma (PAR) and fat pad (MFP) were analyzed for immunoreactive ERα. Rows A to D correspond to a single animal representative for INT−estrogen, INT+estrogen, OVX−estrogen, and OVX+estrogen, respectively. Staining with control serum is also shown in (E). In the PAR column, arrows highlight examples of lightly labeled epithelial cells. In the MFP column, closed arrows indicate examples of ER-positive adipocytes and open arrows ER-positive fibroblasts within dense connective tissue. Bar=50 μm.
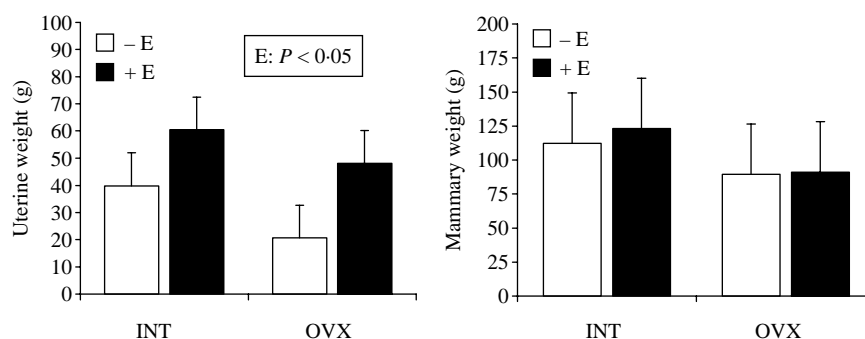
Figure 2 Effect of ovarian status and 17β-estradiol injection on uterine and mammary parenchyma weight. Prepubertal dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 months of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or + estrogen; E). Heifers were slaughtered after 3 days of injection. Uterine weight consists of the cervix and uterine horns. Mammary parenchyma consists of the right hemigland devoid of the mammary fat pad. Bars represent means + S.E.M. The significant effect of 17β-estradiol injection is given in the open rectangle.

later (Purup *et al*. 1993, Berry *et al*. 2003*a*), but this effect was reduced when the OVX was performed after 8 weeks of age (Berry *et al*. 2003*b*).

Consistent with a lack of effect on PAR mass, we were also unable to detect significant effects of OVX on dynamic measurements such as BrdU incorporation by mammary epithelial cells and IGF-I expression in the PAR and MFP compartments. These results are in contrast with those of Berry *et al*. (2003*a*, *b*), where OVX reduced both epithelial BrdU incorporation and PAR IGF-I expression. Again, this discrepancy likely relates to their use of earlier OVX (1–3 months versus 4·6 months in our study) and a longer period of time between OVX and tissue collection (3–5 months versus 30 days in our study). Taken together, these data suggest that effects of endogenous estrogen are greater in the first 2 months of life than at later prepubertal ages. In support of this hypothesis, we recently showed that mammary epithelial BrdU incorporation was highest in ∼2-month heifers and declined steadily as they progress towards puberty (Ellis & Capuco 2002; MJ Meyer, AV Capuco, DA Ross, LM Lintault and ME Van Amburgh, unpublished observations). Obviously, the age-dependent effect of OVX on epithelial cell proliferation and gene expression will need to be examined in future studies.

In contrast, a 3-day period of estrogen administration caused a threefold increase in epithelial BrdU incorporation irrespective of ovarian status, similar to effects previously reported by others in intact prepubertal heifers (Capuco *et al*. 2002). In parallel with increased proliferation, estrogen administration stimulated IGF-I transcript copy number by 1·4-fold in the PAR and 3·6-fold in the MFP. As bovine epithelial cells do not express IGF-I (Berry *et al*. 2003*a*), the stimulation of IGF-I mRNA in the PAR must occur in ER–positive stromal cells. We have recently detected such cells in the interlobular stromal region of the PAR. Consistent with these data, Plath–Gabler *et al*. (2001) found that the only parenchymal cells displaying

immunoreactive IGF-I signals were adipocytes and fibroblasts. In the case of the MFP, there is little doubt that residing adipocytes and fibroblasts are capable of estrogen-dependent IGF-I production. Adipose tissue compares with liver in terms of IGF-I gene expression (Coleman *et al*. 1994) and we now show that 30% of the adipocytes and fibroblasts present in the bovine MFP contain immunoreactive ERα. Finally, we sought to confirm estrogen responsiveness of the MFP by measuring expression of the PR gene. In rodents, this gene, essential for branching morphogenesis (Brisken *et al*. 1998), is shown to be expressed in an estrogen-dependent manner (Kastner *et al*. 1990). Our data show that estrogen treatment increased PR transcript copy number not only in the PAR, but also in the MFP, confirming the ability of the bovine MFP to mount estrogen responses.
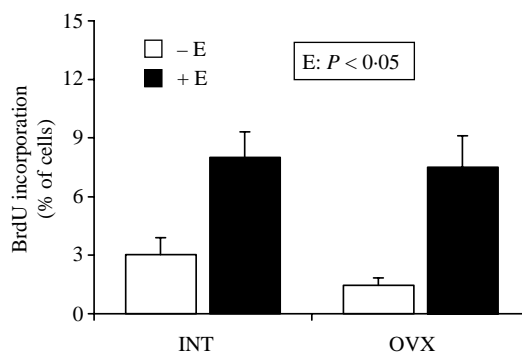
Figure 3 Effect of ovarian status and 17β-estradiol injection on BrdU incorporation in mammary epithelial cell. Sixteen dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 months of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or +estrogen; E). After 3 days of injection, BrdU was administered intravenously and heifers slaughtered 2 h later. Bars represent means ± S.E. The significant effect of β-estradiol injection (−E vs +E) is given in the open rectangle.
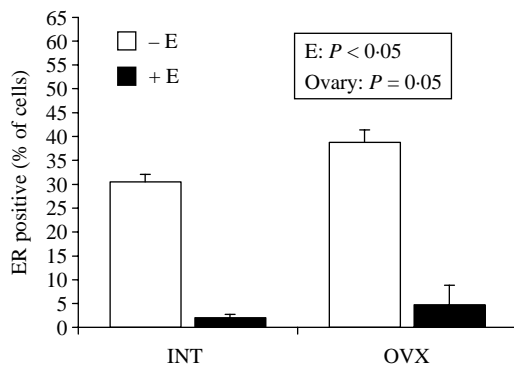
Figure 4 Effect of ovarian status and 17β-estradiol injection on percentage of mammary epithelial cells immunoreactive for estrogen receptor α (ERα). Sixteen dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 months of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or +estrogen; E). Heifers were slaughtered after 3 days of injection. Bars represent means+S.E.M. The significant effects of ovarian status (intact vs, OVX; ovary) and 17β-estradiol injection (−E vs+E) are given in the open rectangle.

Our data indicate that estrogen treatment reduced the number of epithelial cells with immunoreactive ERα signal and that this effect reflects estrogen–dependent inhibition of ERα mRNA abundance. Significantly, this estrogen-dependent downregulation of ERα mRNA did not occur in

the MFP. The ability of the MFP to retain high ERα expression despite the pharmacological estrogen levels may explain its greater IGF-I mRNA response. The physiological consequence of this property may be to blunt the fall of IGF-I production in the MFP as plasma estrogen levels rise during prepubertal development.

Walden *et al.* (1998) showed that estrogen and growth hormone elicited a synergistic IGF-I response in the MFP of ovariectomized and hypophysectomized rats, but not in the SQA. This prompted us to ask whether the bovine MFP differed from its s.c. counterpart. The SQA expressed ERα at only 23% of the level seen in the MFP, and unlike the latter, failed to respond to exogenous estrogen administration by increasing IGF-I or PR expression. To define other possible bases for the lack of estrogen responsiveness in the SQA, we measured the expression levels for another major ER isoform (ERβ) and the orphan receptor most related to ERα (ERRα). Both gene products have been implicated in the estrogen action, with ERβ mediating estrogen effects in the adult rodent mammary gland (Cheng *et al.* 2004) and ERRα attenuating the ability of ERα to mediate the estrogen effects (Zhang & Teng 2001). Our data do not support a role for either of those receptors in explaining differences in estrogen responsiveness as both fat depots had negligible levels of ERβ expression and ERRα abundance did not differ between SQA and MFP. We conclude that variation in ERα

Table 1 Transcript abundance (Transcripts/ng total RNA) of select genes in the mammary parenchyma (PAR), mammary fat pad (MFP), and s.c. adipose tissue (SQA) of prepubertal Holstein heifers assigned to a 2×2 factorial experiment with factors of injection (excipient (−estrogen) or 17β-estradiol (+estrogen)) and ovarian status (intact (INT) or ovariectomized (OVX))

	Treatment[a]					Significance level[b]		
	INT		OVX					
	−Estrogen	+Estrogen	−Estrogen	+Estrogen	S.E.M.	Ovary	Estrogen	Ovary×estrogen
Variable								
	Transcripts/ng total RNA							
PAR								
ERα	990	498	1165	692	124	NS	<0·005	NS
IGF-I	112	188	89	240	24	NS	<0·001	NS
ERRα	21	27	23	27	14	NS	NS	NS
PR	260	714	86	1010	137	NS	<0·001	NS
MFP								
ERα	337	516	401	464	86	NS	NS	NS
IGF-I	177	732	178	558	72	NS	<0·001	NS
ERRα	102	86	87	58	20	NS	NS	NS
PR	19	189	19	94	13	<0·005	<0·001	<0·005
SQA								
ERα	103	68	131	91	12	0·06	<0·01	NS
IGF-I	45	37	44	53	9	NS	NS	NS
ERRα	42	38	34	37	9	NS	NS	NS
PR	13	8	11	11	3	NS	NS	NS

Heifers were slaughtered 30 days after ovariectomy. Factors −estrogen and +estrogen were administered daily for the final 3 days of life.
[a]Sixteen dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 month of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or +estrogen). Heifers were slaughtered after 3 days of injection.
[b]Significance level for ovarian status (Ovary, OVX, or INT), 17β-estradiol injection (estrogen, −estrogen, or +estrogen) or their interaction (Ovary×estrogen). NS, not-significant at P>0·05.
ERα, estrogen receptor alpha; ERRα, estrogen-related receptor alpha; PR, progesterone receptor.
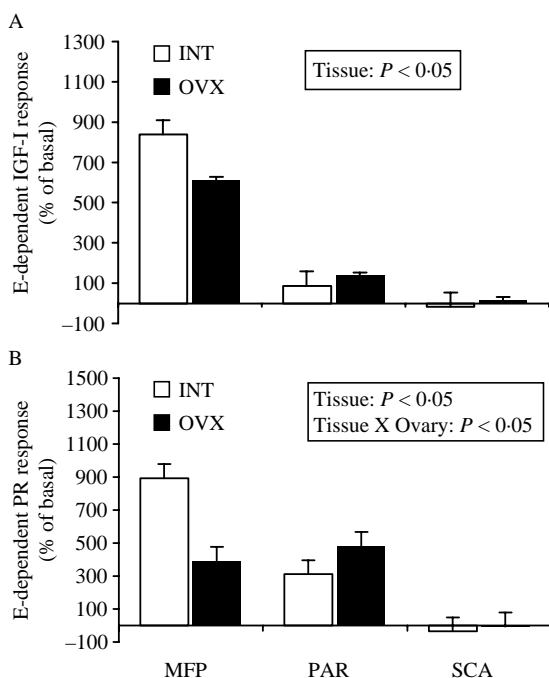
Figure 5 Comparison of estrogen responsiveness across tissues. Sixteen dairy heifers were ovariectomized or left intact (OVX or INT) at 4·6 months of age and injected 30 days later with corn oil or 17β-estradiol (−estrogen or +estrogen; E). Heifers were slaughtered after 3 days of injection. The IGF-I and progesterone receptor (PR) transcript copy numbers were measured in the mammary fat pad (MFP), mammary parenchyma (PAR), and s.c. adipose tissue (SQA) of all heifers. For each gene, the transcript copy number of each estrogen-treated heifer was subtracted from the basal copy number and expressed as percentage of basal copy number (basal = average of all excipient-treated heifers). Bars represent means + S.E.M. The significant effect of ovarian status (ovary), differences among tissues, and their interaction is given in the open rectangle.

expression is likely to explain the different estrogen responsiveness of SQA and MFP.

In summary, the ability of the MFP to increase IGF-I expression is greater than that of its s.c. counterpart or the epithelial-rich PAR. This responsiveness endows the MFP with the ability to coordinate estrogen-mediated prepubertal development in the bovine mammary gland. Obviously, IGF-I is unlikely to be the only functionally important estrogen-dependent growth factor synthesized in the MFP, and for that matter in the PAR as well. Indeed, ER-positive epithelial cells present in the PAR could also secrete unknown mitogens in response to estrogen. The identification of the genes encoding these growth factors and other estrogen-dependent proteins should be advanced considerably by using the recently developed bovine oligonucleotide-based microarrays.

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